"A comprehensive study of Mycoviruses isolated from economically important fungi"

A synopsis of research work proposed to be carried out in pursuance of the requirement for the award of degree of Doctor of Philosophy in Botany (Microbiology)

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INTRODUCTION

Evidences for the viruses of fungi commonly called mycoviruses, was presented as early as 1936 by (Wiebols et al) in yeast and the viruses of higher fungi were first suspected in 1950 when Sinden and Hauser reported a degenerative disease of mushroom. A very severe disease of cultivated mushrooms is widespread and has been shown to be caused by a complex of at least five mycoviruses.

Hollings 1962 reported viruses associated with a die-back disease of cultivated mushrooms and Ellis and Kleinschmidt (1967) reported the first virus of the fast growing fungus, *Penicillium stoloniferum*. Interferon induction was implicated with the presence of ds RNA of this mould.

The early work with *P. stoloniferum* was a definite stimulation whereas in the past detection of a new virus had usually followed the recognition of a disease caused by the virus. It was apparent that viruses of fungi might be found by screening procedures which involve purely physical methods, i.e. partial purification and electron microscopy. A number of laboratories began screening fungus isolates for the presence of virus and met with considerable success.

The fungi represent a heterogeneous assemblage of eukaryotic microorganisms. Fungal metabolism is characteristically heterotrophic, and the vast majority of fungi are filamentous, haploid organisms reproducing either sexually or asexually through spores. Several of the fungi have proven to be excellent experimental systems, and among these are species adapted for formal genetic analysis. Viruses are now recognized as common in fungi and indeed have been reported in species representing each of the major taxonomic classes of the fungi.

The presence of fungal viruses can, therefore, only be revealed through detection of their genomes and (or) the virus like particles (VLP's) in the cell. A few mycoviruses possess single-stranded RNA (ssRNA) or double-stranded DNA (dsDNA) genomes; however the vast majority of mycoviruses have isometric particles of 25– 50 nm in diameter and contain an undivided or segmented double-stranded RNA (dsRNA) genome (Van Regenmortel, et al., 2000).

The isometric mycoviruses are classified into four families including *Totiviridae* (*Totivirus*), *Partitiviridae* (*Partitivirus*), *Chrysoviridae* (*Chrysovirus*) and *Reoviridae* (*Mycoreovirus*). Totiviruses have a linear, uncapped genome of 4.6-7 kbp while the genome of the Partitiviruses, Chrysoviruses and Mycoreoviruses have two, four and 11 or 12 dsRNA segments, respectively (Wickner et al., 2000, Ghabrial et al., 2000, Jiang & Ghabrial 2004, Suzuki et al., 2004). Bruenn (1993) identified eight conserved motifs in RdRps of dsRNA viruses of simple eukaryotes, which

was further found in other dsRNA viruses including mycoviruses (Mertens 2004, Jiang & Ghabrial 2004).

Mycoviruses are widespread and affect many parameters. Their possible effects on the level of toxins and metabolites produced by fungi enhanced their significance in environmental health research.
Clearly, the presence of viruses in fungi adds a new dimension to experimental mycology insofar as these viruses may influence profoundly the metabolism and genetics of the fungal cell.

The presence of some mycoviruses has been associated with particular phenotypic traits, including killer toxin production in *Saccharomyces cerevisiae, Ustilago maydis, Hanseninaspora uvarum* and *Zygosaccharomyces bailii* (Ghabrial, 1994, Schmitt & Neuhausen, 1994), debilitant diseases in *Helminthosporium victoriae* (Nuss & Koltin 1990) and virulence reduction (hypovirulence) in *Sclerotinia sclerotiorum* (Boland 1992), *Leucostoma persoonii* (Hammar et al., 1989), *Nectria radicicola* (Ahn & Lee 2001), *Fusarium graminearum* (Chu et al., 2002), *Cryphonectria parasitica* (Anagnostakis 1982), and so on. Mycoviruses present in mushrooms were also found in *Agaricus bisporus* (Hollings 1962) and *Pleurotus ostreatus* (Goodin et al., 1992). However, in most cases the mycoviruses are latent or cryptic and the fungal hosts harbouring them do not exhibit discernible alterations in their phenotypes (Ghabrial 1994, Ghabrial 1998).
Four dsRNA molecules, with sizes of 4.0, 3.1, 2.7, and 2.2 kbp, were detected in extracts of six (out of 66) isolates of *Fusarium oxysporum* and *F. solani*, in a survey by Kilic and Griffin (1998). No morphological differences were found between dsRNA containing and dsRNA free *F. oxysporum* isolates and the presence of dsRNA did not correlate with hypovirulence in *F. oxysporum* isolates pathogenic to soyabean seedlings. Two dsRNA segments were identified in *F. solani* f. sp. *robiniae* (Nogawa et al., 1993), with sizes of 1.9 and 1.7 kbp that associated with isometric particles of 30 nm diameters. The designation of FusoV was proposed for this virus which was further classified in the genus *Partitivirus* by ICTV (Ghabrial et al., 2000).

Many fungi are pathogenic to higher plants, and viruses are associated with several species of fungi. The pathogenicity of these fungi may be influenced by virus. The interrelationship of virus and fungus with regard to the plant pathology will depend upon whether the virus is a pathogen of the fungus or merely transmitted by the fungus to the higher plant.
Transmission of fungal viruses in general, entails exchange of cytoplasm between fungal cell

lines. The transmission of viruses which infect *A. bisporus* has been extensively studied and reviewed by Hollings and Stones 1969, 1971 and by Dieleman – Van Zaayen 1972. The first experiments to confirm that viruses in fungi
could be transmitted through heterokaryosis were conducted by Lhoas 1970, 1971. These experiments involved genetically marked strains of two fungi, *P. stoloniferum* and *A. niger*.

Different dsRNA profiles were observed in *F. oxysporum* f. sp. *phaseoli* isolates from Italy, Columbia and Poland. Morphological differences were not detected between dsRNA containing and dsRNA-free isolates (Woo et al., 1997).
Various sizes of dsRNAs are also found in *F. graminearum* from barley and maize, some of which had no effect on morphology or virulence (Chu et al., 2002 & 2004). Interestingly, one dsRNA segment was associated with pronounced morphological changes including reduction in mycelial growth, increase in dark orange to red pigmentation, reduced sporulation and virulence.

Double-stranded RNA (dsRNA) are widespread in all classes of plant pathogenic fungi (Buck, 1986). However, in most cases these infections have not been associated with apparent disease symptoms or other phenotypes. In some phytopathogenic fungi, however, dsRNA viruses are associated with reduced virulence (hypovirulence) and other phenotypes such as reduced growth, sporulation, or pigmentation (Nuss and Koltin, 1990). Interestingly in present day extensive studies on the biological roles of dsRNA have been conducted. In *Cryphonectria parasitica*, the chestnut blight fungus, it was found that the conversion of a virulent strain to a hypovirulent one coincided with transmission of dsRNA by hyphal anastomosis (Anagnostakis and Day, 1979).
Trichoderma species are common fungi found in many cultivated and natural soils, and have long been known for their capacity to reduce plant diseases caused by soil-borne fungi (Baker and Cook, 1974; Whipps and Lumsden, 2001) and some have been tested for biological control potential in many field and greenhouse trials as well as commercial formulation available worldwide.

Although mycoviruses are thought to be prevalent among the fungi, the failure of these agents to cause readily discernible changes in infected host cells makes their detection very difficult. Consequently, in lieu of a convenient method, mycoviruses are usually detected by immunological procedures (Lemke, 1979) and also involving physical methods.

Fungi cause catastrophic diseases in all major crops with considerable impact on human lives. For

example, the emergence of the deadly wheat black stem rust fungus is likely to threaten the world's breadbaskets. Application of chemical fungicides is the major method used for control of fungal diseases of economically important crops, especially when resistant cultivars are lacking. To reduce the dependence on fungicides, environmentally friendly alternative methods to control diseases are desirable.

Mycovirus-mediated hypovirulence is a phenomenon in which the virulence of fungal pathogens is reduced or even completely lost as a consequence of virus infection. Hypovirulence is thought to play a role in counterbalancing plant diseases in nature and it was used successfully to control chestnut blight (caused by the fungus *Cryphonectria parasitica*) in Europe. The successful utilization of hypovirulence for biological control of the chestnut blight fungus has attracted much interest and led to the discovery of hypovirulent strains in other fungi (Nuss et *al.*, 2001).

The exploitation of mycovirus for biological control is a promising method and a milestone to be achieved in disease control.

Botrytis cineria is a phytopathogenic fungus, which attacks more than 200 plant species and causes diseases of gray mold, leaf blight, blossom blight, or post-harvest fruit rots In temperate climates, it has become a severe problem on numerous field and greenhouse crops, such as cucumber, tomato, grapes, and strawberry. The presence of single-stranded ssRNA- or dsRNA-mycoviruses in mycelia of *B. cinerea* has been reported . However, pathogenicity tests showed that hypovirulence was not always associated with infection of *B. cinerea* by mycoviruses (Howitt et al., 1995). On the other hand, Castro et al., (2003) found that the strain CCg425 of *B. cinerea* infected by a mycovirus (6.8-kb dsRNA) was less aggressive in infecting leaves of bean than the mycovirus-free strain CKg54 of this pathogen.

Lemaire et al., (1971) indicated that virus infected strains of *O. graminis* produced little or no disease in wheat. When virus infected strains were mixed with normal strains in greenhouse pots and field plots planted with wheat, the disease severity was greatly reduced compared to controls infected with the non virus infected strain of *O. graminis*. This constitutes the first report of biological control of plant pathogen with a mycovirus.

Varying reports related to decreased or increased pathogenicity of fungi by viruses have been reported. In addition to reports on hypovirulence and their possible role in biological control, mycoviruses have also been shown to be responsible for lethal diseases of higher fungi and some plant pathogenic fungi contain mycoviruses. Lapierre et al., (1977) & Lemaire et al., (1978) isolated a mycovirus from *Ophiobolus graminis*, a fungus which causes a severe root rot in wheat.

Therefore, it was concluded that presence of mycovirus increased the pathogenecity in wheat.
Mango malformation is a severe disease prevalent in northern parts (Mallik, 1963; Varma, 1983). Causal agent of this disease of national importance remained a question mark for a number of years. Until Varma (1983) proved that it is caused by *Fusarium moniliforme* var. *subglutinans* but could not get the infection everytime.
Later Gupta (1991) reported that presence of mycovirus in *Fusarium moniliforme* var. *subglutinans* might be responsible for the disease as VLP infected strains of fungus produced malformations in the pathogenicity tests conducted and VLP free isolates resulted in healthy mango seedlings.

Another major role of mycoviruses which has been envisaged is as targets and suppressors of RNA silencing in *Aspergillus* mycoviruses. RNA silencing can function as a virus defence mechanism in a diverse range of eukaryotes, and many viruses are capable of suppressing the silencing machinery targeting them. However, the extent to which this occurs between fungal RNA silencing and mycoviruses is unclear. Here, three *Aspergillus* dsRNA mycoviruses were partially characterized, and their relationship to RNA silencing was investigated. *Aspergillus* virus 1816 is related to *Agaricus bisporus* white button mushroom virus 1 and suppresses RNA silencing through a mechanism that alters the level of small interfering RNA. *Aspergillus* virus 178 is related to RNA virus L1 of *Gremmeniella abietina* and does not appear to affect RNA silencing. The third virus investigated, *Aspergillus* virus 341, is distantly related to *Sphaeropsis sapinea* RNA virus 2. Detection of mycovirus-derived ssRNA from this mycovirus demonstrates that it is targeted for degradation by the *Aspergillu*s RNA silencing machinery. Thus, the results indicate that *Aspergillus* mycoviruses are both targets and suppressors of RNA silencing. In addition, they suggest that the morphological and physiological changes associated with some mycoviruses could be a result of their antagonistic relationship with RNA silencing. (Hammond et al., 2007).

Limited evidence is available on the direct influence of viruses on fungal host metabolism particularly production of secondary metabolites. Diverse reports related to toxin production (galactosamine) (Buck et al., 1969), associated with the presence or absence of mycoviruses of *P. stonloniferum* are there. (Mackenzie and Adler, 1972).

Although the first reports on mycoviruses date from the 1960's, our knowledge and understanding of mycoviruses is still in its infancy, especially work in India is limited.

Moreover, current mycovirus publications primarily concern economically important fungi such as cultivated mushrooms, yeast, and fungal pathogen of plants.

Therefore, in addition to ongoing researches, work will be planned to characterize virus like particles of selected fungal pathogen in order to restart work in the field of mycovirus research especially in Indian context after a long intermittent period.

REVIEW OF LITERATURE

Virus concept:

A virus is a small infectious agent that can only replicate inside the cells of another organism. The word is from the Latin "virus" referring to poison and other noxious substances, first used in English in 1392. "Virulent", from Latin "virulentus" (poisonous), dates to 1400. A meaning of "agent that causes infectious disease" is first recorded in 1728. The term "virion" is also used to refer to a single infective viral particle. The plural is "viruses".

Viruses are too small to be seen directly with a light microscope. Viruses infect all types of organisms, from animals and plants to bacteria and archaea, fungi. Although there are millions of different types.

There are many viruses which are known to infect fungi and the viruses which are present in fungi have been variously named by different workers. Generally, they are called mycoviruses or fungal viruses but fungal viruses are difficult to isolate and characterize therefore it is referred to as VLP's or virus like particles. Most commonly, these particles are known as viruses of fungi. The term mycophages has also been used by some workers.

Discovery of mycoviruses:

VLP's in cultivated mushroom:

Sinden and Hauser, 1950 suspected VLP's in mushrooms when they reported the die back disease of cultivated mushrooms. The symptoms of the disease were a marked decrease in production of mushroom, distorted morphology and premature deterioration of mushroom tissue. Other investigations include (Gandy 1959; Gandy 1960; Kneebone et al., 1962; Storey et al., 1959). Many investigators reported similar but it was Sinden who first suggested that the disease might be because of virus. Therefore, disease of commercial mushroom led to the first observation of mycovirus by electron microscopy (Gandy et al., 1962). Hollings also reported viruses in association with diseased mushroom (Hollings 1962; Hollings 1965; Hollings et al., 1969). Between 1962 to 1965, evidence for viruses in *Agaricus bisporus* were accumulated.

VLP's in species of Penicillium:

The discovery of mycoviruses in Penicillium species were specifically studied in two species i.e. *P. stonloniferum and P. funiculosum* (Powell et al., 1952; Shope 1953). Ellis and Kleinschmidt

1967 demonstrated through electron microscopy that an active antiviral fraction from *P. stonloniferum* contained polyhedral virus particles. Lampson et al., 1967 reported that the interferon inducer from *P. funiculosum* was dsRNA. The viral nature of dsRNA was confirmed by Banks etal., 1968. Mycoviruses were later discovered in many species of Penicillium (Banks et al., 1969; Hollings and Stone 1971; Lemke and Ness 1970; Moffitt and Lister 1973). Mycoviruses of *Penicillium chryosogenum* (Banks et al., 1971; Bozarth et al., 1971; Buck et al., 1971; Cox et al., 1970; Lemke et al., 1971; Nas etal., 1973; Volkoff et al., 1972; Yamashita et al., 1973) was the most extensively studied genus. This fungus was basically employed for commercial production of penicillin. Mycoviruses, however, were not initially discovered in *P. chryosogenum* as it was first reported in fast growing fungus, *P. stonloniferum*. Later mycoviruses were reported in many industrial fungus.

Viruses of other fungi:

Mycoviruses have been reported in many species from different genera of fungi. Most of these reports concerning mycoviruses have been based purely on Electron Microscopy.

Fungus	**Reported description**	**Citation**
	Zygomycetes	
Aphelidium sps	Iridescent type (F)	Schnepf et al., 1970
Aphelidium sps	Polyhedral type	Schnepf et al., 1970
Allomyces arbuscula	Isometric type	Khandjian et al., 1977
Choanephora sps	-	0(a)
Mucor aligarensis	Isometric type	Akade'miai' kiado, 2005
Mucor hiemalis	Isometric type	Akade'miai' kiado, 2005
Mucor corticolus	Isometric type	Akade'miai' kiado, 2005
Mucor mucedo	Isometric type	Akade'miai' kiado, 2005
Plasmodiophora brassicae	-	Adler et al., 1976
Rhizopus stolonifer	Polyhedral,40 nm	Papp et al., 2001
R.microsporus	Polyhedral,40 nm	Papp et al., 2001
R. oryzae	Polyhedral,40 nm	Papp et al., 2001
Syncephalastrum sps	-	0(a)
Ascomycetes		
Cryphonectria parasitica	Spherical,50-90 nm	Chen ans Nuss, 1999
Daldinia sps	-	0(a)

Diplocarpon rosae	Isometric,34-32 nm	Bozarth et al., 1972
Gaeumannomyces graminis	Isometric,27-35 nm	Rawlinson et al., 1973
Hypoxylon multiforme	Spherical type	Wyn Jones et al., 1977
Endothia parasitica	Spherical,50-90 nm	Van Alfen, 1982
Neurospora crassa	Isometric,60 nm	Tuveson and Peterson, 1972
Ophiobolus graminis	Isometric,29 nm	Albouy and Lapierre, 1972; Lapierre et al., 1970; Lemaire et al., 1971
Ophiostoma novoulmi	Rod shaped,17 nm	Lapierre et al., 1970
Periconia circinata	Polyhedral,32 nm	Dunkle, 1974
Peziza ostracoderma	Rods 17x 350 nm	Dieleman- van Zaayen, 1967
Saccharomyces cerevisae	Spherical, 40 nm	Volkoff and Walters, 1970
Sclerotinia sclerotioiom	-	Boland, 1992
Septoria nodorum	Isometric 28 nm,38 nm	Newton, 1987
Basidiomycetes		
Agaricus bisporus (virus 1)	Isometric,25 nm	Hollings, 1962; Hollings and Stone, 1971
Agaricus bisporus (virus 2)	Isometric, 29 nm	Hollings, 1962; Hollings and Stone, 1971; Dieleman-van Zaayen and Temmink 1986
Agaricus bisporus (virus 3)	Bacilliform 19x50 nm	Hollings, 1962; Hollings and Stone, 1971; Dieleman-van Zaayen and Temmink 1986
Agaricus bisporus (virus 4)	Isometric, 35 nm	Hollings, 1962; Hollings and Stone, 1971; Dieleman-van Zaayen and Temmink 1986
Agaricus bisporus (virus 5)	Isometric,50 nm	Hollings, 1962; Hollings and Stone, 1971
Agaricus bisporus	Rods 17x350 nm	Dieleman-van Zaayen, 1967

Agaricus bisporus (LIV)	Isometric	Ellis and Kleinschmidt, 1967
Agrocube aegerita	-	Barros and Labarere, 1990
Boletus sps	Isometric,50 nm	Holling, 1962
Hypholoma sps	-	0(a)
Laccaria amethystine	Isometric,28 nm	Blattny and Kratik, 1968
Laccaria laccata	Isometric,28 nm	Blattny and Kratik, 1968
Polyporus sps	-	0(a)
Tilletiopsis sps	Isometric,40 nm	0(c)
Tilletia indica	Flexous, rod shaped	Beek et al.,1994
Trichothecium roseum	Hexagonal,35 nm	George et al.,1981
Ustilago maydis	Isometric 41 nm	Wood and Bozarth,1972
Fungi imperfecti		
Alternaria tenuis	Isometric,30-40 nm	Lau J.Reid and Kim, 1981
Alternaria tenuis	Spherical, polyhedral 33-44 nm	Lau J.Reid and Kim, 1981
Arthrobotrys	-	0 (b)
Aspergillus flavus	Isometric,30 nm	Mackenzie and Adler,1972
Aspergillus flavus	Spherical,27-30 nm	Wood et al., 1973
Aspergillus foetidus (IMI 41871)	Isometric,40-42 nm	Banks et al., 1970; Ratti and Buck, 1972
Aspergillus foetidus S (IMI 41871)	Isometric,40 nm	Ratti and Buck, 1972
Aspergillus foetidus F (IMI 41871)	Isometric, 40 nm	Ratti and Buck, 1972
Aspergillus glaucus	Isometric, 25 nm	Hollings, 1962
Aspergillus niger (IMI 146891)	Isometric,40-42 nm	Banks et al., 1970
Botrytis cinerea	Isometric, 33 nm	Lemke and Ness, 1970
Candida albicans	Spherical,12,18,30 nm	Mehta et al.,1982
Candida curvata	Linear, 35 nm	Matte et al., 1990
Candida tropicalis	Spherical,80-120 nm	Sharzei et al., 2007
Cephalosporium acremonium	-	Day and Ellis, 1971
Chrysosporium sps.	-	0 (b)
Fusarium moniliforme	Isometric 40 nm	Gupta, 1991
F. oxysporum	Isometric, 35 nm	Sharzei et al.,2007
F. poae	Isometric, 30 nm	Mehta et al., 1982
F. solani	Isometric, 30 nm	Nogawa et al.,1993

Gliocladium sps	-	0 (a)
Gliomastic sps	-	0(b)
Helminthosporium maydis	Isometric,40 nm	Bozarth et al., 1972
Helminthosporium victoriae	Polyhedral,35-40 nm	Linderberg, 1959; Sanderlin and Ghabrial 1978
Kloeckera sps	-	0 (a)
Mycogone perniciosa	Isometric,42 nm	Lapierre et al., 1972
Mycogone perniciosa	Rods 18x120 nm	Lapierre et al., 1972
Paecilomyces sps	-	0 (a)
Penicillium brevicumpactum	Isometric,40 nm	Wood et al., 1971
Penicillium brevicumpactum	Spherical, 40 nm	Wood et al., 1971
P.chrysogenum ATCC 9480, NRRL1951,B25,X1612,Q176,WIS48701, WIS5120C,BRL 700	Isometric,35 nm	Lemke and Ness, 1970
P. chrysogenum ATCC 9480	Isometric,40 nm	Lemke and Ness, 1970
P. chrysogenum ATCC 9480	Polyhedral,40 nm	Nash et al., 1972
P. chrysogenum NRRL 1951	Isometric,35 nm	Wood and Bozarth 1972
P. citrinum	Isometric,40-50 nm	Banks, 1968
P. cyaneofulvum CMI 58138	Isometric, 32 nm	Banks et al., 1969
P.funiculosum	Isometric,25-30 nm	Banks, 1968
P.funiculosum	Polyhedral	Banks, 1968
P. multicolour	Isometric, 32-34 nm	Ellis and Kleinschmidt, 1967
P.notatum	Isometric, 25 nm	Ellis and Kleinschmidt, 1967
P.stoloniferum ATCC 14586	Isometric,25-30 nm	Bozarth et al., 1971
P.stoloniferum F ATCC 14586	Isometric, 32-34 nm	Planterose et al., 1970
P.stoloniferum S ATCC 14586	Isometric, 32-34 nm	Planterose et al., 1970
P.variabile	Isometric, 40-50 nm	Banks, 1968
Piricularia oryzae	Isometric, 32 nm	Yamashita et al., 1971
Piricularia oryzae	Isometric , 36 nm	Yamashita et al., 1971
Rhizoctonia solani	Linear, 33 nm	Finkler et al., 1988;Finkler et al 1985
Sclerotium cepivorum	Isometric, 30 nm	Albouy and Lapierre, 1972; Lapierre and Faivre-Amoit, 1970
Spicaria	-	0(b)

Verticillium dahlia	Isometric	Cao et al., 2011

0, Unpublished results of (a) Merfyn Richards, Beecham Research Laboratories, Betchworth, England; (b) Rodger Crosse, Glaxo Research Ltd., Stoke Poges, Bucks, England; (c) R.F. Bozarth and H.A. Wood.

Physical properties of Mycoviruses:

A few mycoviruses possess single-stranded RNA (ssRNA) or double-stranded DNA (dsDNA) genomes, however the vast majority of mycoviruses have isometric particles of 25– 50 nm in diameter and contain an undivided or segmented double-stranded RNA (dsRNA) genome (Van Regenmortel, et al., 2000). The nucleic acid extracted from viruses infecting many fungi (Table 2) has proven to be dsRNA.

Table 2: Physical properties of nucleic acids from mycoviruses:

Mycoviruses	**Morphology**	**Diameter**	**References**
Penicillium chryosogenum	Polyhedral	35 nm	Buck et al., 1971
P. brevicompactum	Polyhedral	36-40 nm	Wood et al., 1971
P. stonloniferum	Polyhedral	34 nm	Bozarth et al., 1970
P. cyaneofulvum	Polyhedral	32.5 nm	Banks et al., 1969
Ustilago maydis	Spherical	41 nm	Wood and Bozarth 1973
Periconia circinata	Polyhedral	32 nm	Dunkle 1974
Aspergillus foetidus	Polyhedral	33-37 nm	Ratti and Buck 1972
Drechslera sps.	Isometric	35 nm	Hurley S. Shepherd et al. (1990)
Candida curvata	Isometric	35 nm	Matte et al., (1990)
Endomyces magnusii	Isometric	43 nm	Pospisek et al., (1994)
Epichloë festucae	Isometric	50 nm	Iñigo Zabalgogeazcoa et al. (1998)
Fusarium oxysporum sps. *melonis*	Isometric	35 nm	Sharzei et al., (2007)
Pleurotus ostreatus	Isometric	35 nm	Kim et al., (2008)
Geotrichum candidaum	Isodiametric	40 nm	Mor et al., 1984

Single stranded RNA has been isolated from *Neurospora crassa* (Kuntzel et al., 1973). Tavantzis et al, (1980) isolated ssRNA mycoviruses were isolated from *Agaricus bisporus.* Yu et al., (2003) reported a novel single stranded RNA mycovirus in *Pleurotus ostreatus*. A mycovirus, named oyster mushroom spherical virus (OMSV), was isolated from cultivated oyster mushrooms with a severe epidemic of oyster mushroom Die – back disease.

Effects of mycoviruses on their host:

Interferon induction:

Early reports on interferon induction suggested that host mediated interference of viral synthesis followed from the discovery of interferon (Issacs and Lindenmann, 1957). They suggested that RNA is an inducer. Two fungal products, statolon from *P. stonloniferum* and helenine from *P. funiculosum* were found to have the capacity to induce interferon in test animals. Since then dsRNA of mycoviruses isolated from *P. chryosogenum* (Wood and Bozarth, 1972; Banks 1969; Buck et al., 1971; Bozarth and Wood 1971), *P. cyaneofulvum* (Banks 1969), *A. foetidus* (Banks 1970), and *P. funiculosum* (Banks 1968) has been shown to induce interferon. The therapeutic potential of dsRNA has been extensively investigated. Evidence reported that free viral dsRNA is more active as inducer of interferon than intact particles (Nemes et al., 1969). Kleinschmidt 1972 reported cellular immunity against several viruses by inducing interferon.

Killer phenomenon:

The killer system in yeast, *Saccharomyces cerevisae* was initially recognized as a genetic phenomenon (Makowar and Bevan, 1963). Three phenotypes were described i.e. Killer, sensitive and neutral. Some strains of *U. Maydis*, a smut fungus of corn, produce a toxic protein lethal only to sensitive strains of the fungus (Day and Anagnostakis 1972; Hankin and Puhalla 1971). Analysis of inheritance of killer and immune function has established that the killer phenotype is determined by an extra chromosomal factor that is dependent on nuclear genes for its maintenance. Many scientists recognised these nuclear genes associated with killer system and its suppression (Bevan and Somer, 1969; Bevan et al., 1973; Fink and Style; Vodkin et al., 1974, Wickner and Leibowitz 1976 a, b).

Bussey and Skipper 1976 reported that killer factor from *S. cerevisae* kills not only sensitive *S. cerevisae* but also the pathogenic yeast *Torulopsis glaberata* which involves membrane damage. *Candida, Debaryomyces*, *Pichia* and *Torulopsis* are some genera of yeast in which killer systems

were found (Phillipskirk and Young, 1975). The potential use of killer toxin in control of smut fungi have been screened for sensitivity to *U. maydis* toxins (Koltin and Day, 1975). An example is the killer system described in *Pichia anómala,* which shows toxic activity against a wide variety of nonrelated microorganisms, such as hyphomycetes and bacteria, including important opportunist pathogens, such as *Candida albicans* (Polonelli et al., 1986; Polonelli et al., 1989; Turchetti and Buzzini, 2003). In the biotechnological área, the use of killer strains to eliminate undesirable microorganisms in industrial fermentations or in food preservation has been suggested (Sulo and Michalcakova, 1992; Sulo et al., 1992; Lowes et al., 2000).

Lytic plaque formation:

Plaque formation is not a routine formation but it is found in some species of fungi. Lytic plaque formation are associated with viruses of fungi. The virus of *P. chryosogenum* has been extensively studied in this regard (Albouy and Lapierre 1971; Lemke et al., 1973). It is also occur in three species of Penicillium (Lemke et al., 1973; Borre et al., 1971). Plaque formation is correlated with an increase in extracellular virus titer. It is also occur in the strains of *Schizophyllum commune* (Koltin et al., 1973). This formation is transmissible through heterokaryosis. Mehta et al., 1981 also reported lytic plaque formation in *Candida albicans.*

Effects of mycoviruses on physiology:

A significant change was observed in growth rate and virulence between isolates that contained dsRNA and those that did not (Bottacin et al., 1993). Pyung and Yong (2000) suggests that in some fungi, ds RNA mycovirus infection causes distinct morphological and physiological changes, including toxin production (Magliani et al., 1997; Varga et al., 1994), cytological alterations of cellular organelles (Newhouse et al., 1983), virulence associated traits such as growth rate (Boland 1992), sporulation (Bottacin et al., 1994), pigmentation (Anagnostakis 1979) and enzymatic activities (Rigling and Van Alfen 1993). The strain of Fusarium graminearum in which mycovirus was present had pronounced morphological changes, including reduction in mucelial growth, increased pigmentation, reduced virulence and decreased production of trichothecene mycotoxins.

Transmission:

The exchange of cytoplasm between fungal cell lines is called transmission of fungal viruses. Viral transmissions are achieved by fungi through many processes like heterokaryosis, spores,

protoplast fusion, etc. Earlier attempts to transmit viruses to several higher plants have failed (Hager 1966, Hollings M. 1962).

The transmission of viruses which infect *A. bisporus* has been extensively studied and reviewed by Hollings and Stones 1969, 1971 and by Dieleman – Van Zaayen 1972. The first experiment to confirm that viruses in fungi could be transmitted through heterokaryosis was conducted by Lhoas 1970, 1971. These experiments involved genetically marked strains of two fungi, *P.stoloniferum* and *A.niger*. Viral transmission through heterokayosis has been studied in different species of fungi, for example, *Ustilago maydis* (Wood and Bozarth 1973), *Schizophyllum commune* (Koltin et al., 1973), *Gaeumannomyces graminis* (Lemaire et al., 1971), *Penicillium claviforme* (Metitiri and Zachariah 1972), *Aspergillus glaucus* (Jinks 1959), *Podospora anserine* (Marcou 1961).

Conidial transmission tests were conducted by Penny et al., 1986.

Transmissions of viruses through spores were studied in few fungi for example *A. bisporus* (Dieleman van Zaayen, 1974). Spores are an important factor in dissemination and maintenance of viruses in fungal isolates reported by Yamashita et al., 1973.

The reports suggested that uninfected protoplasts of *Penicillium stonloniferum* incubated in the presence of purified virus became infected (Lhoas 1971). Diepeningen et al., (1998) suggested intra and interspecies virus transfer in *Aspergilli* via protoplast fusion.

Virulence and hypovirulence of mycoviruses:

Increased pathogenecity:

Varying reports related to decreased or increased pathogenicity of fungi by viruses have been reported. In addition to reports on hypovirulence and their possible role in biological control Mycoviruses have also been shown to be responsible for lethal diseases of higher fungi and some plant pathogenic fungi contain mycoviruses. Lapierre et al., (1970) & Lemaire et al., (1978) isolated a mycovirus from *Ophiobolus graminis*, a fungus which causes a severe root rot in wheat.

Therefore, it was concluded that presence of mycovirus increased the pathogenecity in wheat.

Since all the dsRNA patterns were recovered from natural field infection it seemed unlikely that any of them would have profound effects in either reducing or enhancing pathogenecity unless there were interactions between dsRNA pattern or between dsRNA pattern and nuclear genes. The pathogenecity tests were carried out on corn smut from Connecticut by Day, 1981. Hashiba et al., (1984) reported weakly pathogenic isolates of *R. solani*, 1668 RI- 1, 1271 RI64 and 1272 RI –

1 which showed abnormally slow growth contained plasmids, but pathogenic isolates 1668, 1271 and 1272 showing normal growth, contained no detectable plasmid DNA.

Mango malformation is a severe disease prevalent in northern parts (Mallik, 1963; Varma, 1983). Causal agent of this disease of national importance remained a question mark for a number of years. Until Varma (1983) proved that it is caused by *Fusarium moniliforme* var. *subglutinans* but could not get the infection everytime.

Later Gupta (1991) reported that presence of mycovirus in *Fusarium moniliforme* var. *subglutinans* might be responsible for the disease as VLP infected strains of fungus produced malformations in the pathogenicity tests conducted and VLP free isolates resulted in healthy mango seedlings.

Decreased pathogenecity or Mycoviruses as biocontrol agent:

Fungi cause catastrophic diseases in all major crops with considerable impact on human lives. For example, the recent re-emergence of the deadly wheat black stem rust fungus is likely to threaten the world's breadbaskets. Application of chemical fungicides is the major method used for control of fungal diseases of economically important crops, especially when resistant cultivars are lacking. To reduce the dependence on fungicides, environmentally friendly alternative methods to control diseases are desirable.

Mycovirus-mediated hypovirulence is a phenomenon in which the virulence of fungal pathogens is reduced or even completely lost as a consequence of virus infection. Hypovirulence is thought to play a role in counterbalancing plant diseases in nature and it was used successfully to control chestnut blight (caused by the fungus *Cryphonectria parasitica*) in Europe. The successful utilization of hypovirulence for biological control of the chestnut blight fungus has attracted much interest and led to the discovery of hypovirulent strains in other fungi (Nuss, 2001). The exploitation for mycovirus for biological control is a promising method and a milestone to be achieved in disease control.

Botrytis cineria is a phytopathogenic fungus, which attacks more than 200 plant species and causes diseases of gray mold, leaf blight, blossom blight, or post-harvest fruit rots In temperate climates, it has become a severe problem on numerous field and greenhouse crops, such as cucumber, tomato, grapes, and strawberry. The presence of single-stranded (ss) RNA- or dsRNA-mycoviruses in mycelia of *B. cinerea* has been reported. However, pathogenicity tests showed that hypovirulence was not always associated with infection of *B. cinerea* by mycoviruses (Howitt

et al.1995) On the other hand, Castro et al. (2003) found that the strain CCg425 of *B. cinerea* infected by a mycovirus (6.8-kb dsRNA) was less aggressive in infecting leaves of bean than the mycovirus-free strain CKg54 of this pathogen.

Lemaire et al., (1971) indicated that virus infected strains of *O.graminis* produced little or no disease in wheat. When virus infected strains were mixed with normal strains in greenhouse pots and field plots planted with wheat, the disease severity was greatly reduced compared to controls infected with the non virus infected strain of *O.graminis*. This constitutes the first report of biological control of plant pathogen with a mycovirus.

Characterization of mycoviruses of different genera of fungi.

Matte et al., (1989) characterized dsRNA molecule of 1.55 um in length and three major protein component have MW of 75700, 53000, 27000 daltons from *Candida curvata.* Bottaicin et al., (1993) also characterized a mycovirus from *Chalara elegans* of 2.8 kb. Castro et al., (2003) characterized mycovirus from *Botytis cineria*. The virus was located in the fungus cytoplasm as free particles of approximately 28 nm in diameter. It possesses a single double stranded genome of 1.8 kbp encapsidated within an isometric protein coat whose component is a polypeptide of 68 kDa. Pyung and Yong (2000) have characterized four dsRNA's of size 6, 5, 2.5, 1.5 kbp in 24 out of 81 strains of *Nectria radicicola*, the causal fungus of ginseng root rot. Valverde et al., (2009) reported the molecular characterization of dsRNA viruses infecting plants and fungi. He has found a partitivirus and a totivirus infecting Jalape pepper, and tomato respectively. A 7.5 kb dsRNA's from 13 of 286 field strains of *F. graminearum* isolated from maize by Yeon et al., (2002). Similar report has been found by Darissa et al., (2011) purified a mycovirus from *F. graminearum* of 2.4 to 3.5 kbp. Four dsRNAs, dsRNA 1 (3554 bp), dsRNA 2 (3250 bp), dsRNA 3 (3074 bp) and dsRNA 4 (3043 bp), were detected in rice blast fungus, *Magnaporthe oryzae by* Urayama et al., (2010)

First reports on mycoviruses:

Varga et al., (1998) reported double-stranded RNA mycoviruses in species of *Aspergillus* sections *Circumdati* and *Fumigati.* Isolates belonging to *Aspergillus sections Fumigati, Candidi, Clavati* and *Circumdati* were tested for the presence of double-stranded RNA (dsRNA) genomes and this is the first report on the detection of naturally occurring dsRNAs in *Aspergillus* species that are able to reproduce sexually.

Virus like particles in *Chrysosporium* species reported for the first time and *C. albicans* reported for the first time from India. Both are human pathogenic fungi. (Sharma et al., 2011)

Indian contribution in the field of mycovirus research:

Information available is scanty.

Maheshwari and Gupta (1973) reported antiviral agents from *Aspergillus flavus.* Morphology, transmission and ultrastructure of VLP's in *Helminthosporium sps.* was described. (Misra et al., 1979).

Hexagonal VLP's 45 nm in diameter were detected in Himachal strain of *Trichothecium roseum.* (George et al., 1981)

Tewari and Singh 1984, 1985 isolated isometric VLP's from *Agaricus bisporus*.

Later, Gupta (1990) conducted detailed studies on viruses of fungi. VLP's of *R. solani* were purified and characterized. Also various morphological types of VLP's were detected in 4 species of 4 genera of fungi out of 13 species belonging to 7 genera of fungi, along with various other detailed studies (unpublished data).

Advanced research pertaining to field of mycoviruses:

A novel alcohol oxidase/RNA-binding protein with affinity for mycovirus double-stranded RNA were isolated from the filamentous fungus *Helminthosporium (Cochliobolus)victoriae* by Soldevila and Ghabrial, (2000). They have cloned and sequenced a novel alcohol oxidase (Hv-p68) from the filamentous fungus *Helminthosporium (Cochliobolus) victoriae* that copurifies with mycoviral double-stranded RNAs. Preisig et al., (2000) studied the novel RNA mycovirus in a hypovirulent isolate of the plant pathogen *Diaporthe ambigua*. In this study, they established the complete cDNA sequence of this ds RNA, which represents a replicative form of a positive – strand RNA virus that they have named *D. ambigua* RNA virus (DaRV). The nucleotide sequence of the genome is 4113 bp and has a GC contentes of 53%.

Diepeningen et al., (2006) studied the dynamics of dsRNA mycoviruses in black *Aspergillus* populations.

A mutualistic association between a fungal endophyte and a tropical panic grass allows both organisms to grow at high soil temperatures and this was shown by Márquez et al., (2007). He characterized a virus from this fungus that is involved in the mutualistic interaction. Fungal isolates cured of the virus are unable to confer heat tolerance, but heat tolerance is restored after the virus is reintroduced. The virus-infected fungus confers heat tolerance not only to its native monocot host but also to a eudicot host, which suggests that the underlying mechanism involves pathways conserved between these two groups of plants.

It was reported that *Aspergillus* mycoviruses are targets and suppressors of RNA silencing. In

addition, they suggest that the morphological and physiological changes associated with some mycoviruses could be a result of their antagonistic relationship with RNA silencing (Hammond et al., 2008)

Polymorphism of viral dsRNA in *Xanthophyllomyces dendrorhous* strains isolated from different geographic areas were studied by Baeza et al., (2009). In this work, the characterization and genetic relationships among dsRNA elements were determined in strains representatives of almost all regions of the earth where *X. dendrorhous* have been isolated.

A novel mycovirus that is related to the human pathogen hepatitis E virus and rubi-like viruses were reported by Liu et al., (2009).

The complete nucleotide sequences of four dsRNAs associated with a new chrysovirus infecting *Aspergillus fumigatus* were reported by Jamal et al., (2010).

OBJECTIVES

1) Screening and indexing of pathogenic fungal cultures for the presence of virus like particles through Electron Microscopy.

2) Attempt to cure/ eliminate virus like particles from the selected pathogenic fungal cultures.

3) To correlate pathogenecity of selected plant pathogenic fungal culture with presence or absence of VLP's.

4) To conduct transmission studies.

5) To study the effect of virus infection on fungal morphology and growth of selected fungal culture.

6) Purification and characterization of virus like particles of selected pathogenic fungal culture.

METHODOLOGY

1) Screening and indexing of various pathogenic fungal cultures for the presence of virus like particles through electron microscopy.

- Human pathogenic fungi.
- Food spoiling fungi.
- Plant pathogenic fungi.

Will be screened for the presence of virus like particles (VLP's)

Mediums used:-

SABOURAUD'S DEXTROSE AGAR (SDA) MEDIUM:

INGREDIENTS	AMOUNT
Peptone	10gm/l
Dextrose	40gm/l
Agar	20gm/l
Distilled water	1000ml
Chloramphenicol	0.025gm/l
pH	5.6

CZAPEK AGAR MEDIUM:-

INGREDIENTS	AMOUNT
Sodium nitrate	2 gm/l
Potassium dihydrogen phosphate	1 gm/l
Magnesium sulphate	0.5 gm/l
Potassium chloride	0.01 gm/l
Sucrose	30 gm/l
Agar	20 gm/l
Distilled water	1000ml
Chloramphenicol	0.025 gm/l

All media will be autoclaved at 15lb pressure for 20 minutes in pre-sterilized conical flask. After autoclaving fungal cultures will picked up from the stock culture with the help of sterilized needle and transferred to the petridishes containing culture medium in aseptic condition.

Mass propagation of mycelia:

For obtaining mycelial mats of different fungal isolates mass production in liquid culture medium will be carried out in 250 ml conical flask. Approximately 100 ml of media will be poured in flask respectively. Each flask will be tightly plugged by cotton plugs, wrapped with butter paper. These will sterilized by autoclaving for 20 minutes at 15lb pressure. Autoclaved flasks will be inoculated with a 4-5 mm piece of agar culture under aseptic conditions in laminar flow. Inoculated flasks will be incubated for 15-20 days at 27^{o}C in incubator.

Composition of liquid culture medium:

SABOURAUD'S DEXTROSE AGAR (SDA) BROTH:-

INGREDIENTS	AMOUNT
Peptone	10gm/l
Dextrose	40gm/l
Distilled water	1000ml

Chloramphenicol	0.025gm/l
pH	5.6

CZAPEK BROTH:-

INGREDIENTS	AMOUNT
Sodium nitrate	2 gm/l
Potassium dihydrogen phosphate	1 gm/l
Magnesium sulphate	0.5 gm/l
Potassium chloride	0.01 gm/l
Sucrose	30 gm/l
Distilled water	1000ml
Chloramphenicol	0.025 gm/l

Harvest of mycelia:

Mycelial mats will be harvested by filtration through cheese cloth and washed twice with distilled water to remove media. Mycelial mats will squeeze dry in double folds of blotting paper. Before further treatment, fresh weight of fungal mycelium will be recorded.

Disruption of mycelia:

Mycelial disruption will be achieved through two methods:-

Disruption of frozen mycelium:-

Harvested mycelial mats will be blotted dry in double folds of blotting paper and wrapped in cellophane sheets. These will be kept in deep freeze -20^{o} C for 48 hours. Frozen mycelium will powdered in a sterile mortar with pestle.

a) With abrasive: 5 ml of 0.1M phosphate buffer, pH 7.0 per gram weight of mycelium will be added. After thorough grinding with carborundum (abrasive), material will be filtered and squeezed through double layered cheese cloth. Re extraction of mycelium will be done. Homogenate will be centrifuged at 5,000 rpm for 45 minutes to remove cell debris.

b) Without abrasive: 5 ml of 0.1 M phosphate buffer, pH 7.0, per gram wet weight of mycelium

will be added. After thorough grinding, material will be filtered and squeezed through double layered cheese cloth. Re extraction of mycelium will be done. Homogenate will be centrifuged at 5,000 rpm for 45 minutes to remove cell debris.

Identification of VLP's through electron microscopy.

Supernatant obtained after low speed (5,000 rpm for 45 min.) centrifugation of homogenate will be applied to carbon coated grids, stained with uranyl acetate and rinsed twice with distilled water. Presence of VLP's will be checked by examining grids under electron microscope at an instrument magnification of X 20,000. On the basis of screening and VLP's detection, fungal isolates will be selected for further studies.

2) Attempt to cure/ eliminate VLP's from the selected infected fungal cultures.

(i) Through hyphal tip isolations:

For hyphal tip isolations of the selected VLP infected fungal isolates. Water agar will be autoclaved at 15 lb pressure for 20 mins. This will be poured in sterilized petri plates. After solidification, selected fungal isolates will be plated on medium under aseptic conditions.

These will be grown for 3 to 5 days at 25°C and examined under a dissecting microscope. Single hyphal tips will be excised at 1 mm to 2 mm distance using a sterile stainless steel needle and transferred to petri plates with 14 ml of sabouraud's agar medium under aseptic conditions. Four tips will be inoculated on each petri plate. After their growth, peripheral tips will be again transferred on sabouraud's agar medium. After four successive repetitive tip transfers at four days interval, finally hyphae from periphery will be transferred to respective liquid culture medium for mass culture.

(ii) Cycloheximide treatment:

The VLP infected isolate will be treated with cycloheximide treatment. In this, cycloheximide, an inhibitor of protein synthesis (Fink and Styles, 1972), will be added to SDA at 10, 20, 50, 100, 200 µg/ml. Mycelial plugs of the isolate will be transferred to these media and the plates will be incubated at room temperature in the dark for 2 weeks. Then small plugs will be removed from the margin of the colonies, transferred to SDA broth and finally examined for the presence of VLP's.

(iii) Thermotherapy:

Stock cultures of selected fungal isolates will be transferred to agar medium in test tubes and incubated for 2 weeks at 25 °C (Nair, 1973).

Immediate heat treatment:

Mycelial discs 4-5 mm in diameter from stock cultures will be transferred to petri plates containing 14 ml of SDA medium. After inoculation, petri plates will be placed at once in an incubator at 35 °C. Two replications per fungal isolate will be kept. These will be grown for three weeks at 35 °C. Radial growth will be measured for 12 days. After 3 weeks of thermal treatment, mycelial discs from edge of the colonies will be transferred to SDA medium's slants in test tubes. Linear growth will measured at two day interval for 10 days. Four replicates per fungal isolate will be kept. Tip transfers of heat treated cultures in test tubes will be incubated at 25 °C. Linear growth will measure along the surface of the agar slope 9 cm long, obtained by sloping test tubes, containing 10 ml of medium. Controls will be untreated virus infected cultures, kept throughout at 25 °C. Presence of VLP will be checked after heat treatment.

3) To correlate pathogenecity of selected plant pathogenic fungal cultures with presence or absence of VLP's.

(i) Pathogenecity test:

To determine if the dsRNAs affect virulence of the host fungus, pathogenecity test of *Fusarium moniliforme* var. *subglutinans* isolates i.e. VLP infected and VLP free will be done on mango seedlings grown in pots. Healthy seedlings will be inoculated with *Fusarium* isolates giving a cut at the shoot tip. After placing a inoculums, the

inoculated tip will be tied with a cotton swab and covered with polythene. Cotton swab will be kept moist for 10 to 15 days. The experiment will be carried out in three replications (Gupta S. 1991).

(ii) To test whether mycoviruses can be used as biocontrol agents:

Prepare extracts of VLP free isolate and VLP infected isolate. Spray the extract on the diseased infected part of leaf and observe the changes.

4) To conduct transmission studies.

(i) Transmission of viruses through hyphal anastomosis.

Anastomosis between infected and free culture will be demonstrated by placing mycelia plugs of each taken from the advancing margin of young cultures, 2 cm apart on a petri plate filled with a layer of PDA. The plates will be incubated at 25 °C until the hyphae of the isolates intermingled. Transmission experiments involved the coinoculation of diseased and healthy cultures on opposite sides of PDA plates. Diseased isolate will be inoculated prior to healthy isolate and incubated for 7 days. 4 mm diameter plugs will be taken from the side of the plate inoculated with healthy culture and another inoculum will be taken from the side with diseased culture for each of three replicates and transferred to PDA.

(ii) Transmission of viruses through protoplast fusion.

Young mycelia will be prepared and incubated for 3 hours at 30 °C with 1 M NH_4Cl containing 5 mg/ml of driselase, 2 mg of novozym and 8 mg/ml of lysing enzyme. Protoplast will be harvested by centrifugation at 2544 x g at 4 °C for 10 mins, washed twice with STC (1.2 M sorbitol, 10 mM tris HCl, pH 7.5, 50 mM $CaCl_2$), and suspended in 300 ul of MMC buffer (0.6 M mannitol, 10 mM MOPS, pH 7 and 10 mM $CaCl_2$). Equal volumes of the two protoplast suspensions (100 ul of 1x107 protoplasts/ ml) will be mixed and placed on ice for 30 mins. After this, 500 ul of PEG solution (60% PEG 3350, 10 mM MOPS, pH 7 and 10 mM $CaCl_2$) will be added to the protoplast suspension, the mixture will be incubated at 20 °C for 20 mins. Protoplast fusants will be regenerated in 700 ul of potato dextrose broth for 7 days in the dark, plated on 15 ml of YCDA (0.1 % yeast extract, 0.1 % casein hydrolysate, 0.5 % glucose and 1.5 % agar), and then selected on PDA containing 50 ug/ml of hygromycin B and 50 ug/ ml of geneticin. This will be confirmed by EM or agarose gel electrophoresis.

5) To study the effect of virus infection on fungal morphology and growth of selected fungal culture.

Following cultures will be inoculated onto SDA medium on petri plates and incubated at 37 °C. the colony diameters of the isolates will be measured every 24 hours over a period of 5 days and growth rate per time interval used to calculate the average growth rate per day. All experiments will be performed in triplicate. To assess biomass production equal amount of mycelium of the isolates will be inoculated into 100 ml flask containing SDA broth and incubated at 37 °C on a

rotator shaker (130 rpm) over a period of 5 days. The mycelium from individual cultures will be harvested daily by filtration through cheesecloth and the pellets dried at 37 °C until their weights will be constant and biomass produced per time interval used to calculate the average growth rate per day. All experiments will be performed in triplicate. T – test will be used to analyse all of the data for significant differences in growth rate and biomass production.

6) Purification and Characterization of VLP's of selected fungal isolates.

(a) Following protocol will be followed for isolation and purification of VLP's of selected fungal isolates.

(i) Harvesting: Selected infected fungal culture will be mass propagated in liquid culture medium in flasks. Extraction will be done by homogenization of frozen mycelium followed by grinding in mortar and pestle. Extraction buffer will be 0.05 M phosphate buffer, pH 7.1. Add w/v 0.05 M NaCl + 0.001 EDTA + 0.05 M $MgSO_4$.

(ii) Clarification:

By low speed centrifugation:

Homogenates will be clarified by employing low speed centrifugation; 8,000 – 10,000 rpm for 20 to 30 minutes.

(iii) Concentration:

Polyethylene glycol (PEG) precipitation:

PEG 6000 (10% w/v) will be added to clarified extract along with 0.5 M NaCl and mixed thoroughly by stirring on magnetic stirrer in cold for 2.5 hours. Host proteins along with VLP's will be precipitated by centrifugation at 10,000 rpm for 20 mins. The pellet will suspended in PO_4 buffer 0.05 M, pH 7.1. Finally, the particles will be precipitated by giving a cycle of low (10,000 rpm x 20 mins.) speed centrifugation. Final pellet will be dissolved in 1 ml PO_4 buffer.

Quantitative assay of the virus:-

For quantitative estimation, the absorbance at 260 nm of the partially purified preparation will be measured spectrophotometrically and extinction coefficient $E\ 260^{0.1\%}_{1\ cm}$ will be calculated by the formula:

$E\ 260^{0.1\%}_{1\ cm} = 1.531 + 0.205\ (RNA\%)$

The virus titre will be calculated by the formula:

Virus titre in purified preparation (mg/ml) = $\frac{A\ 260}{E\ 260\ ^{0.1\%}_{1\ cm}}$ x dilution

Virus titre in host (mg/gm) = $\frac{A260 \text{ x dilution x } 1}{E\ 260\ ^{0.1\%}_{1\ cm} \quad \text{gm fresh wt.}}$

UV absorption spectra will be measured in UV / VIS spectrophotometer. A quartz cuvette containing 1 ml suspension buffer will be first placed in the sample chamber and the instrument will be adjusted to read 'zero' absorbance. Another cuvette with partially purified virus suspension will be then placed in the chamber and absorbance will be recorded. Sample will be scanned over the wavelength ranging from 230 – 300 nm.

(b) Extraction of nucleic acid.

(i) From VLP's of selected fungal isolate:

Single phase phenol - SDS method of Diener and Schneider (1968**)** will be followed. One volume of virus suspension in 0.02 M PO_4 buffer, pH 7 with one volume of reagent [Reagent = 0.4 ml of phenol (1 volume of water + 5 volumes 90% phenol) + 1.5 ml of 3 percent SDS in 0.02 M PO4 buffer, pH 7 with 0.01 M EDTA] will be incubated for 15 min at 4°C. Aqueous phase will be collected after running a low speed centrifuge at 7000 rpm for 10 mins. Nucleic acid will be precipitated with ethanol. For 1 ml of virus preparation, 2 volumes of cold absolute ethanol will be added. This will be stored at -20 °C for 1 hour. Low speed centrifugation 6000 rpm for 10 mins. will be given. Pellet will be suspended in 1 X TBE buffer (Tris – Borate – EDTA buffer). Ethanol – precipitation and resuspension will be repeated twice.

(ii) From mycelium of selected fungal isolate:

Method of Sansing et al., (1973) and Detroy et al., (1974) will be adopted. Ten grams of frozen mycelium of fungal isolate will homogenized in 0.05 M PO_4 buffer, pH 7.2 (0.5 ml/gm). Two volumes of cold absolute ethanol will be added to homogenate and keep it at -20 °C for 2 hour. Precipitate will be sedimented by low speed centrifugation (8,000 rpm x 10 min.). Dissolved precipitate in 0.2 M sodium acetate and equal volume of aqueous 90 percent phenol containing 0.1 percent (w/v) 8- hydroxyquinoline. This will be shaken for 20 mins. and low speed

centrifugation, 8000 rpm x 20 mins. will be given.

Aqueous phase will be separated and nuceic acid will precipitated from 0.2 M sodium acetate with 2 volumes cold ethanol by repeated precipitation (4 times). Final precipitate will be dissolved in minimal volume of resuspension buffer i.e., 1xTBE.

(iii) Extraction of nucleic acids and purification of dsRNA by cellulose chromatography: (Valverde, 1990):

The procedure is as follows:

1. 5 ml of 0.1 M phosphate buffer, pH 7.0 per gram weight of mycelium will be added. After thorough grinding with carborundum (abrasive), material will be filtered and squeezed through double layered cheese cloth. Re extraction of mycelium will be done. Homogenate will be centrifuged at 5,000 rpm for 45 minutes to remove cell debris.
2. Add 1.0 ml of 10% SDS, 0.5 ml of bentonite (from a 2% aqueous suspension), and 9.0 ml of 1X STE-saturated phenol to the homogenate and shake it well for 30 min.
3. Centrifuge tubes at 8,000 g for 15 min. Withdraw 10.0 ml of the upper aqueous phase and place it in a 50 ml centrifuge tube. (If 10.0 ml is not available, adjust to 10.0 ml by adding 1 X STE.)
4. Add 2.1 ml of 95% ethanol to each tube containing 10.0 ml of sample and mix well. (Samples can be stored overnight at 4 °C.)
5. Weigh two 1.0-g portions of celIulose (Whatman CF- 11) per sample and place them in 50 ml tubes. Add 25 ml of 1 X STE containing ethanol, 16.0% v/v.
6. Prepare two columns, using for each the barrel of a 20-ml plastic syringe plugged with a disk of Miracloth paper or glass wool. Mix the cellulose suspensions well, pour them into the columns, and allow the STE to drain through.
7. Add the sample {must be at room temperature) to one column and let it drain completely. Discard the liquid from the column. Flush the column with 40 ml of 1 X STE containing ethanol, 16.0% v/v. Keep refilling the column until the entire buffer is used. Let it drain completely, and discard the liquid.
8. Add 2.5 ml of 1 X STE and let it drain completelv. Add 10.0 ml of 1 X STE, but this time collect 10.0 ml in 50-ml centrifuge tubes. Add 2.1 ml of 95% ethanol, then repeat step 7, using the second column. Go to step 9.

9. Add 2.5 ml of 1 X STE and let it drain. Add 6.0 ml of 1 X STE and collect 6.0 ml in a 50 ml centrifuge tube. Add 0.5 ml of 3.0 M sodium acetate (pH 5.5) and 20.0 ml of 95% ethanol to each sample. Store for at least 2 hour at -20 °C to precipitate the nucleic acid.

10. Centrifuge samples at 8,000g for 25 mins. Pour off the ethanol and place the tubes upside down to drain for about 15 mins. Add 200 µl of EG buffer to each tube and mix well to resuspend the nucleic acid. Store samples (indefinitely) at -20 °C.

Electrophoresis of nucleic acid can be done in a variety of ways, but it is usually performed in 6% polyacrylamide gels or in 1.0-1.5% agarose gels. The appropriate volume of nucleic acid extract to load on the gel varies according to the virus. Normally, 30 - 50 µl is needed.

(c) Ascertaining nature of nucleic acid.

(i) Sensitivity to nucleases:

Nucleic acid samples will be treated with DNAse (15 µg/ml) in 0.1 M sodium acetate containing 0.005M $MgSO_4$, pH 5 and incubated at 25 °C for 30 mins. Nucleic acid sample will be treated with RNAse in standard saline citrate (SSC) 1 N (0.15 M NaCl + 0.015 M sodium citrate, pH 7.2) for 30 mins at 37 °C (0.05 ml of sample + 0.0005 ml of nucleases) (Marino et al., 1976; Koltin and Day, 1976a; Hicks and Haughton, 1986). 12 µl samples will be loaded on agarose gel and electrophoresis will be done.

(ii) Sensitivity to mycoviral RNA in high and low salt to RNAse.

Samples treated with ribonuclease in high (1SSC) and low (0.01 SSC) salt conditions will be determined after incubating samples at 25 °C for 30 min (Bozarth, 1977; Kim and Bozarth, 1985). After the treatment, agarose gel electrophoresis will be done. The DNA marker which will be used, 1 kb DNA ladder provided from Hi media.

Agarose gel electrophoresis:

0.5 gm of agarose in 50 ml TBE buffer in 250 ml flask

↓

Microwave it for about 1 minute

↓

Leave it to cool for 5 minutes

↓

Add 1 µl of EtBr (10mg/ml)

↓

Seal the tray with tape

↓

Pour the gel in tank and insert the comb

↓

Leave to set for 30 minutes

↓

Pour TBE buffer into the gel tank to submerge the gel to 2-5 mm depth

↓

Add sample + bromophenol blue in the wells

↓

Connect the terminals and allow to run the gel at 80 V for 4 hours

(iii) Thermomelting

Hyperchromicity of nucleic acid will be determined. The absorbance of nucleic acid in 0.01 SSC at 260 nm will be determined at 40, 50, 60, 70, 80, 90 °C (Bozarth, 1977; Kim and Bozarth, 1985).

d) Extraction and characterization of protein by SDS-PAGE.

The polypeptide composition of viral particles will be analysed by 10% SDS and protein bands will be visualized by staining the gels with coomassie blue.

Reagents used:

5 X Sample buffer:

Chemicals	Amount
SDS	10 % w/v
Beta mercapto – ethanol	10mM
Glycerol	20 % v/v
Tris HCl, pH 6.8	0.2 M
Bromophenol blue	0.05 % w/v

1 X Running buffer:

Chemicals	Amount
Tris HCL	25 mM
Glycine	200 mM
SDS	0.1% w/v

1 X Running gel solution:

Chemicals	Amount
Distilled water	15.3 ml
1.5 M Tris HCl, pH 8.8	7.5 ml
20% (w/v) SDS	0.15 ml
Acrylamide/Bisacryamide (30%/ 0.8% w/v)	6.9 ml
10% (w/v) ammonium persulphate (APS)	0.15 ml
TEMED	0.02 ml

Stacking gel solution (4% Acrylamide):

Chemicals	Amount
Distilled water	3.075 ml
1.5 M Tris HCl, pH 8.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bisacryamide (30%/ 0.8% w/v)	0.67 ml
10% (w/v) ammonium persulphate (APS)	0.025 ml
TEMED	0.005 ml

Pouring the gels:

Mix the ingredients and pour the solution quickly into the gel casting form – be sure to leave a some room for the stacking gel. Usually leave about 2 cms below the bottom of the comb for the stacking gel. Can be done by inserting the comb into the dry form, and marking a region below the comb for the height of the stacker. Look for the bubble and remove them, then layer the top of the gel with water saturated butanol or very carefully with water. This will remove bubbles at the top of the gel and will ensure this part does not dry out. Wait for about 30 min for the gel to polymerize completely.

While waiting mix the re agent for the stacking gel, but leave out the APS and TEMED until ready to pour this gel, stacking gels will polymerize more quickly than desired. When the running gel is polymerized wash out the butanol completely or the stacker may separate from the gel. Mix in the polymerizing re agents and pour the stacking gel on top of the running gel. Insert combs trying not to get bubbles stuck underneath and allow another 30 min to 1 hrs for complete polymerization. Gels are ready.

Preparing the Sample:

Mix protein 4:1 with sample buffer. Heat the sample. Boiling for 5 to 10 min.

Running the Gel:

Clamp in gel and fill both buffer chambers with gel running buffer according to the specific apparatus. Pipet the sample into the gel adjusting the volume according to the amount of protein in the sample. Use 5 ug of Coomassie blue stain. Be sure to include a lane with molecular weight standards. Now attach the power leads and run the gel until the blue dye front reaches the bottom. Run at 250 V constant. Remove the gel from the power supply and process further. Visualize the proteins using Coomassie brilliant blue, silver stain or any of the other protein stains.

SIGNIFICANCE

Mycoviruses are ubiquitous, found in all major groups of fungi. Fungal viruses were first found in mushroom in the 1950's. Thus fungal virology is a relatively new field that had been underappreciated. However, recent findings of a growing number of novel mycoviruses have expanded the knowledge of virus epidemiology, diversity and evolution. New insights into viral replication, gene expression strategies, virion structures, virus/host and virus/virus interactions, and the induction of RNA silencing antiviral defense responses have resulted from recent studies on mycoviruses. While many viruses cause asymptomatic infections in their host fungi, some influence fungal host physiology. Some mycoviruses infections have a toxin mediated killing capacity.

A virus was recently shown to confer heat tolerance to the endophytic fungus host and, in turn, the plant host of the endophyte, giving an example of a novel type of mutualistic interactions. From the perspectives of applied sciences, mycoviruses have the potential to be used as virological (biological) control agents when they reduce virulence of their phytopathogenic host fungi.

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*Originals not seen